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miR-29b upregulates miR-195 by targeting *DNMT3B* in tongue squamous cell carcinoma

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Abstract MicroRNAs play important roles in the development and progression of various cancers, including tongue squamous cell carcinoma (TSCC). miR-29b and miR-195 have been reported to be tumor suppressors in TSCC. Here, we investigated the expression of miR-29b and miR-195 and their relationship in TSCC. Our data showed that miR-29b and miR-195 were significantly downregulated in TSCC compared with their matched nonmalignant tissues in 60 paired samples. The level of miR-29b was positively correlated with that of miR-195 in TSCC and the matched nonmalignant tissues. Moreover, miR-29b overexpression induced the demethylation of CpG islands upstream of miR-195 via targeting *DNMT3B*, leading to the upregulation of miR-195 in TSCC cell lines. Following *DNMT3B* silencing, the expression of miR-195 was increased and the methylation of CpG islands upstream of miR-195 was reduced. Although overexpression of miR-29b alone significantly increased miR-195 expression, co-transfection of miR-29b with DNMT3B resulted in no change in miR-195 expression. Taken together, our results demonstrated that miR-29b could upregulate miR-195 by directly targeting DNMT3B in TSCC. The interaction between miR-29b and miR-195 might provide new insights in developing novel therapeutic approaches of TSCC.

Keywords miRNA · miR-29b · miR-195 · DNMT3B · Tongue squamous cell carcinoma

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1 Introduction

Tongue squamous cell carcinoma (TSCC) is the most common type of oral cancer and has a particularly poor prognosis due to its invasive nature [1]. Although the etiology of TSCC is not fully understood, aberrant epigenetic regulation contributes substantially to the onset and progression of TSCC as well as many other cancers [2, 3]. Among the mechanisms of epigenetic regulation, noncoding RNAs [4] and DNA methylation [5] are key determinants in the pathogenesis of human cancer.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that control target gene expression by messenger RNA (mRNA) degradation or translation inhibition [6]. Aberrant expression of certain miRNAs is associated with squamous cell carcinoma; they can function as oncogenes or tumor suppressor genes depending on the mRNAs targets they regulate [7–9]. For instance, miR-7/184/138/21

have been shown to play critical roles in the development and progression of TSCC [10–13]. We also previously showed that miR-29b and miR-195 are significantly reduced in TSCC [14, 15]. miR-29b functions as a tumor suppressor in many malignant tissues by targeting genes including *MMP2* [16], *CX3CL1* [17], *Sp1* [14, 18], and *c-FOS* [19]. miR-29b represents an example of "epi-miRNAs" since it targets epigenetic effectors like DNMT3A/3B, leading to the re-activation of oncosuppressive genes [20–24]. Meanwhile, aberrant miR-195 expression is observed in multiple types of cancer [25–27]. Aberrant expression of miRNA could, in part, be caused by epigenetic regulators that control their expression [28, 29]. Particularly, the expression of miR-195 is silenced by hypermethylation of the upstream cytosine-phosphate-guanine (CpG) island [30]. Considering that mir-29b targets DNMT3B and the expression of miR-195 is influenced by DNA methylation, we postulated that miR-29b could modulate miR-195 expression by regulating DNMT3B activity in TSCC. Informed consents was received from each patient.

In the present study, we demonstrated that miR-29b upregulates miR-195 by directly targeting DNMT3B in TSCC. Our findings present a novel modulatory mechanism of miRNA biogenesis and provide insight into the treatment of TSCC.

2 Materials and methods

2.1 Human tissue specimens

Paired primary TSCC samples and adjacent histologically normal tissues were obtained from 60 patients after obtaining informed consent. None of the patients received treatment prior to radical surgical treatment. Tumor tissues and matched nonmalignant tissues at least 1.5 cm distal to the tumor margins were snap-frozen in liquid nitrogen and stored at −80 °C until use; part of these specimens were also used in our previous studies [14, 15]. This study was approved by the Institutional Ethics Committee of Peking University School of Stomatology (Beijing, China).

2.2 RNA isolation and quantitative stem-loop reverse transcription PCR (qRT-PCR)

Total RNAs were isolated from the tumor and normal tissue samples using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Oligo-dT-primed cDNA was obtained using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). Quantitative PCR was conducted at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s in an ABI 7500 real-time PCR machine (Applied Biosystems). The relative expression of miR-29b and miR-195 was calculated by the $2^{-\triangle\triangle Ct}$ method. The qRT-PCR primers used to amplify miR-29b, miR-195, *DNMT3B*, *U6* (internal control for miRNAs), and *β-actin* (internal control for mRNAs) are listed in Table 1.

2.3 Vector construction and luciferase reporter assay

The expression vector pcDNA3.1-miR-29, harboring miR-29b precursor, was generated as described previously [14]. A firefly luciferase reporter plasmid including wild-type and the 3′-untranslated region (UTR) of mutant *DNMT3B* was also created [31]. TSCC cells grown in 48-well plates were co-transfected with 400 ng of pcDNA3.1-miR-29 and 40 ng of the firefly luciferase reporter plasmid including either the wild-type or mutant 3′-UTR of *DNMT3B*, and 4 ng of pRL-TK, a plasmid expressing *Renilla* luciferase (Promega, Madison, USA). Luciferase activity was measured 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega).

2.4 Cell culture and transfection

The human tongue cancer cell lines SCC-15 and CAL27 were purchased from the American Type Culture Collection (Manassas, USA). All tongue cancer cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum containing 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at 37 °C in a humidified atmosphere with 5 % CO_2. SCC-15 and CAL27 cells were seeded onto 6-well plates the day before transfection to ensure 80 % confluency at the time of transfection.

2.5 5-Aza-2-deoxycytidine (5-Aza-dC) treatment

Cells were seeded 24 h prior to treatment with 5 mmol/L 5-Aza-dC (Sigma-Aldrich, St. Louis, USA). After 5 d of treatment, cells were harvested and total RNAs were prepared for qRT-PCR analysis.

2.6 Bisulfite sequencing for DNA methylation analysis

The University of California Santa Cruz database was used to identify CpG islands (CGI) spanning miR-195 genes. Bisulfite sequencing for miR-195 promoter DNA methylation analysis was performed as previously described [30]. DNA was extracted with phenol–chloroform and treated with bisulfite using a CpGenome^{TM} DNA modification kit (Millipore, Billerica, USA) according to the manufacturer's instructions. Bisulfite-converted genomic DNA, in which unmethylated cytosines were converted to uracils, was amplified with specific primers as previously described